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STUDIES ON THE METHODS OF DECALCIFICATION OF BONES

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I. PREFACE

In making histological preparations of bones, teeth, and other hard tissues containing calcium, the decalcification is necessary, which needs much time and trouble, such as repeated renewals of decalcifying reagents. Since 1947, when RICHMAN's school published the electric decalcification method, various attempts have been made to shorten the time required for the decalcification. The present study has been made for the evaluation of such methods. The decalcification is nothing but a chemical reaction through which insoluble calcium salts are rendered soluble by acids. Therefore, it is evident that the velocity of decalcification is proportional to the temperature and the concentration of reagents. However the dissolution of the calcium salts contained in bones proceeds much more slowly as compared with an usual chemical reaction, because they are firmly enclosed by semipermeable membranes. For this reason the acceleration of the decalcification is attempted in various ways, but it should always be done within a certain limit of the temperature as well as of the concentration of reagents in order to avoid the tissue damage causing the impairment of the stainability.

II. THE REASONS WHY THE VELOCITY OF DECALCIFICATION HAS NOT NUMERICALLY BEEN SHOWN.

1) The velocity of decalcification varies according to the kind and age of the animals, and to the parts of a bone (epi-, meta- and diaphysis), or to whether the bone is normal or pathologic.

2) The bones of young animals are readily sectioned in lamellar preparations without being decalcified.

3) Even after complete decalcification it is very difficult to make sections of bones, (i) in case they are hardened by dehydration in alcohol, and (ii) in case they are embedded in paraffin, particularly if they contain the cartillage tissue.

4) The velocity of decalcification varies depending on the surface dimension and the size of the bone specimens.

All of these factors should be taken into consideration in the study of the velocity of decalcification.

III. METHOD, RESULTS AND DISCUSSION

The questions to be answered in the present study have been;

(1) How long does it actually take for the decalcification?

(2) How the decalcification velocity is to be accelerated?

Thus following experiments have been made;

(i) The effects of various kinds of acid on the velocity of decalcification.
(ii) The effects of the concentrations of the acids as reagents.
(iii) The effects of the temperatures of the acids on the velocity of decalcification.
(iv) The effect of the mixture of various kinds of acid or of some annexes on the velocity of decalcification.
(v) The electric decalcification.
(vi) The lower-pressure method and the lower-pressure warming method.
(vii) The effects of the ion-exchange-resin on the velocity of decalcification.
(viii) E. D. T. A. (ethylen-diamine-tetraacetic acid) method, i. e. versenate method.
(ix) The effects of the ultra-sound wave on the velocity of decalcification.

As a method roughly to determine the decalcification velocity, I take a bone piece, which has been dipped in the reagents only on one side and covered with paraffin on the other, and measure the thickness of the layer of bone which has been decalcified and become sectionable with a microtome. For the experiment, a piece of the cortex (0.5×1cm) excized from the shaft of a femoral bone of an adult dog is used and its periosteal side is dipped in the decalcifying reagents, while the remaining sides are covered with paraffin.

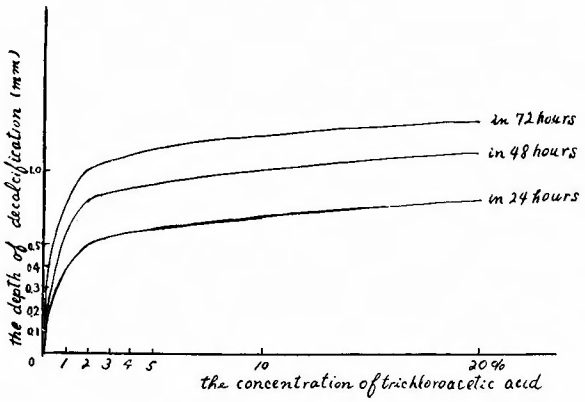
The results of the experiments are as follows.

1) The effects of various kinds of acid on the velocity of decalcification. The velocity of decalcification is compared among the hydrochloric, nitric, and trichloroacetic acids of the same normality. The hydrochloric acid of 0.2%, the nitric acid of 0.4%, the trichloroacetic acid of 1% are of the same normality, viz. 0.06 N; their decalcification velocities are all around 0.4mm in 24 hours under 16°C. The hydrochloric acid of 1.5%, the nitric acid of 2.5% and the trichloroacetic acid of 7% are all 0.4 N, and capable of decalcifying the bone piece under the same condition up to $0.6 \sim 0.7$mm. The fact means that the decalcification velocity is dependent on the concentration of acids, and not on their kinds. Exceptions are such acids as sulfuric acid which form hardly soluble substances. But in case of acids of sufficiently high normality to manifest the proper actions of their own, they cause the impairment of the stainability and the destruction of tissues in variable degrees according to the kinds of acids, even if they are of the same normality. In my experiences, the hydrochloric acid over 10%, the nitric acid over 5% and the trichloroacetic acid over 10%, seem to impair the stainability and to damage the bone tissue, though they decalcify the tissue completely in 48 hours under $30 \sim 35°$ C.

2) The effects of the concentration of strong acids on decalcification: 1, 2, 5, 10, and 20% solutions of the trichloroacetic acid are used as reagents. The results are shown in Fig. 1 and summarized as follows.

(1) The decalcification velocity is higher in case of the reagents of higher concentration.

Fig. I



But beyond a certain limit the increase of velocity does not follow the increase of concentration.

(2) As the decalcification progresses and the decalcified zone becomes thicker, the velocity of decalcification becomes slower, even if the reagents are supplied anew each day.

(3) After the decalcified zone has reached a certain width, the velocity of decalcification remains almost constant regardless of the concentration of the chemicals used. Thus it may be assumed that even in the cases where the complete decalcification needs more than several days, the renewal of the reagents is not necessary and the reagents need not be of high concentration.

3) The effect of the temperature of the reagents on the velocity of decalcification :

The velocity of decalcification is accelerated by warming the reagents just as in any chemical reaction. The experiments : The time required for the complete decalcification of a femoral bone of an adult rabbit has been determined by using the trichloroacetic acid solutions of 5, 10 and 20% respectively as reagents. The results are as follows :

	5%	10%	20%
37°C	16 hours	8 hours	5 and half hours
25°C	20 hours	11 hours	7 hours
8°C	37 hours	27 hours	14 hours

Thus the decalcification is definitely accelerated by warming. But it may be erroneous to attempt to obtain by calculation the exact rate of the acceleration of decalcification per 1°C of warming.

4) The effect of the mixture of various acids or of some annexes on the velocity of decalcification :

The mixture of various acids, or of acids and alcohols, with or without some additional annexes have long been used for the acceleration of decalcification. But as mentioned above, the velocity of decalcification relates to the mol-concentration (molarity) of the reagents, and therefore such mixtures and annexes do not seem to be effective in accelerating the decalcification velocity.

The results of my experiment in this connection are summarized as follows.

(1) In case of the mixture of two strong acids, the velocity of decalcification is proportional to the sum total of the mol-concentration of the two acids.

(2) In case of the mixture of strong and weak acids, the velocity is decided by the mol-concentration of the strong acid.

(3) In case of reagents containing alcohol, the velocity of decalcification

decreases as the amount of alcohol increases.

(4) Annexes (phloroglucin, mercury bichloride, ammonium chloride etc.) have no effects either in accelerating the decalcification, or in preventing the reduction of the stainability due to the chemical destruction of tissues.

5) The electric decalcification:

This was introduced by RICHMAN's school in 1947 as an epoch-making method, and proved to be useful by many followers. It is believed that the principle of this method is in the forcible absorption by the anode of calcium ions in the osseous tissue. But this idea is obviously wrong for the following reasons.

(1) Calcium ions easily pass through a semipermeable animal membrane. For example, when a burned bone, which is wrapped with the bladder of a dog, is dipped in acids, calcium ions are seen to pass through the membrane.

(2) In the reexamination of the original method of RICHMAN, there is certainly some difference between the concentration of calcium ions around the anode and that around the cathode, but the difference is not so great as to suggest the acceleration of the velocity of decalcificaton. There is no trace of out-salting of calcium at the anode.

(3) In considering from the standpoint of the mobility and the velocity of ions, H-ion moves with the velocity 6 times as quick as that of Ca-ion, and 2H-ions come out as H_2 in the air, i, e. in the outside of the field of chemical reaction, whereas Ca remains in the solution as ions without being salted out at the electric pole. Though the chemical reaction $2H^+ \rightarrow 1/2H_2$ goes on, the succeessive moving of Ca-ions toward the anode is unlikely to occur; almost all of the electrons are carried by H^+.

(4) Furthermore, as LISTER and others stated, the fact, that the decacification can be promoted by an alternative current, gives a strong counterevidence against the forced pulling out of Ca-ions.

(5) Regardless of the position of bones in the RICHMAN's electric tub, the decalcification takes place in the same way.

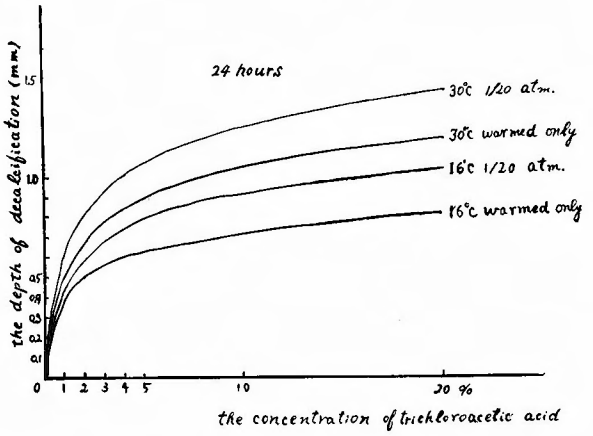
(6) The velocity of decalcification is equal under the same temperature, no matter whether the current is passed or not.

In short, the effect of the electric decalcification is solely ascribed to the JOULE's heat due to current.

6) The lower-pressure and lower-pressure-warming methods:

The carbon-dioxyde gas is generated as the result of the action of acids on the calcium bicarbonate in the bone. Small bubbles of the gas become gradually larger and come out of the bone. This phenomenon occurs not only on the surface of the bone but in the canalicular system in the osseous substance. Thus the reaction of the osseous substance to the decalcifying reagents is suspended so long as the bubbles exist. The lower-pressure-method is aiming at the acceleration of decalcification as the result of the removal of the bubbles by letting them swell more quickly. Furthermore, if the reagents are warmed, the decalcification would be completed much more quickly. Using the water stream pump in order to decrease the pressure, I could get the pressure around 1/20 atm. The result of this experiment

Fig. Ⅱ



is schematically shown in Fig. Ⅱ, and summarized as follows.

(1) The decalcification can be accelerated by decreasing the pressure. The decalcification under lower pressure proceeds 30% further in 24 hours than under normal pressure and the same temperature.

(2) The acceleration of decacification under lower pressure tends to be promoted, though not remarkably, with the reagents of higher concentration.

(3) By warming in addition to decreasing pressure, the decalcification can be further accelerated.

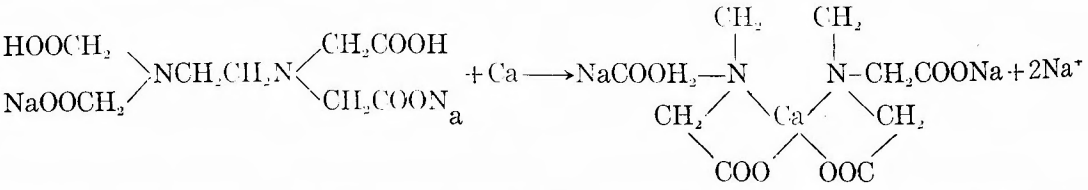
(4) The rate of acceleration is higher at the temperature of 30°C and 1 atm than at 16°C and 1/20 atm.

7) The effects of the ion-exchange-resin on the velocity of decalcification :

In considering that the ion-exchange-resin removes Ca-ions in solution and liberates H-ions, the resin may be expected to be effective for decalcification. In my experiment, however, it has been proved that the only effect of the ion-exchange-resin is to prevent the pH of the decalcifying reagents from elevating, so that it saves the trouble of renewing the solution. But there is no good reason to believe that the ion-exchange-resin is effective for the acceleration of decalcification. But if one insists to use it, H-type of the resin would be preferable, which should before use be dipped in 5% hydrochloric acid and washed with aqua distillate.

8) The effects of versenate (E. D. T. A.) on the velocity of decalcification :

The versenate is a decalcifying reagent of neutral or weak acidic reaction and, for this reason, may be attractive. The process of decalcification can be illustrated in the following formula.



As disadvantages (i) the reaction proceeds very slowly and (ii) since the molecular weight of Ca and that of the combining E. D. T. A. are in the proportion of 1 to around 7, the amount of E. D. T. A. required is great and too much expensive for the ordinary use. But it seems convenient for the staining of neurofibrils or phosphatases which is impossible after decalcification with acids. A brief comment will be made on the use of E. D. T. A. The commonest of E. D. T. A. is 2 sodium type, the water solution of which is weak acidic, while 3 sodium type is

weak alkaline. By mixing the equal amount of these two types, the decalcification can be done in the neutral medium. By using water solution, the femur of a cat is decalcified in one week to such a degree as to be cut with a microtome. The Figs. 2 and 3 show the preparations stained by BIELSCHOWSKY's method for nerve fibers after E. D. T. A. – decalcification, magnification being 40 and 200 times respectively.

9) The effects of the ultrasound wave on the velocity of decalcification :

The decalcification is a slowly progressing chemical reaction, since the decalcifying reagents can not easily reach the inside of a bone because of the presence of semipermeable animal membranes. An experiment has been made in order to know the possible influences of the dispersing action of the ultrasound wave on the decalcification velocity. Using a generator of 2,000 M. A. and 50 KC with a 3 mm crystal board attatched to it, and regulating the machine not to heat the solution higher than 36°C, the irradiation has been made on the bone dipped in 5% trichloroacetic acid. No influences on the velocity of decalcification is observed under these conditions. But this may be due to the short duration of the irradiation (about 1 hour).

Ⅳ. THE IMPAIRMENT OF THE STAINABILITY AND THE DESTRUCTION OF TISSUES.

As have been mentioned in the preceding paragraphs, the factors which are essential for the acceleration of the velocity of decalcification are the following three, viz.;

1) The temperature of the decalcifying reagents.
2) The concentration of H-ion, in strong acids, and the pH in weak acids.
3) The atmospheric pressure acting on the surface of the decalcifying reagents.

These factors should be reconsidered from the points of the impairment of the stainability and the destruction of tissues. No remarkable impairment is seen when the decalcification is done under lower concentration and higher temperature or under higher concentration and lower temperature. But as both the temperature and the concentration become higher, the impairment becomes gradually apparent. The combinedation of the temperature, concentration and time is important as the cause of the impairment of the stainability and the destruction of tissues. Also the structural difference of various bones plays another role. According to my experience, if the decalcification is completed in less than 48 hours under 35°C and with use of 8% hydrochloric, 5% nitric or 10% trichloroacetic acid, neither the impairment of the stainability nor the destruction of tissue is observed. One may be anxious about the destruction of tissues due to the abrupt expansion of bubbles at the time of the lowering of pressure, but no such evidence is actually seen in the microscopic preparations. But in case of a long bone, the formation of CO_2 bubbles in the bone marrow can hinder the succeeding dehydration with alcohol. The preventive measures for this is either the preliminary longitudinal bisection of the bone or the addition of weak alkali after the completion of decalcification in order to absorb bubbles.

V. THE METHOD OF CHOICE OF DECALCIFICATION

From the results of my experiments, I should like to recommend the following "lowered-pressure-warming method". In this method the bone specimen is fixed beforehand for 24 hours in formalin. The too long fixation tends to slow down the velocity. The specimen is cut in thin pieces of less than 5 mm thickness. 10% trichloroacetic acid is used as the decalcifying chemical. Strong acids such as hydrochloric or sulfuric acids possibly impair the stainability if they are applied for a long time. The trichloroacetic acid of more than 10% should not be used, because it does not accelerate the decalcification velocity any more, but is apt to cause the increase of destruction. Generally speaking, it is not necessary to change the reagents in case of a bone piece smaller than the tip of the little finger. The optimal temperature of the chemical is 30° to 35°C, and the destruction of tissues occurs more intensely under the higher temperature. The pressure is reduced as low as possible with a water stream pump (down to about 1/20 atm). For this purpose, it is convenient to use the thermostatic tub of 30° to 35°C with a water stream pump attatched to it. The decalcification rate by this method is 1/3 to 1/6 as compared with that by the ordinary method.

VI. SUMMARY

1. The decalcification velocity is influenced not by the variety of acids, but by the normality or pH of acids, the temperature of decalcificating chemicals, and by the atmospheric pressure.

2. The mixture of various kinds of acid and that of some annexes are not effective in accelerating the decalcification velocity, and in preventing either the destruction of tissues or the reduction of the stainability. The decalcifying velocity is slowed down by adding alcohol.

3. The ion-exchange-resin has no essential effects on the decalcification velocity, though it saves the trouble of renewing the chemicals.

4. The effect of the electric decalcification is ascribed solely to the JOULE's heat due to the electric current.

5. The E. D. T. A. (versenate) method of decalcification is useful for some specific stainings, but not necessary for the ordinary haematoxyline-eosin staining.

6. The effect of the ultra-sound wave on the decalcification velocity does not seem to be remarkable.

7. To the impairment of the stainability and the destruction of tissues are related the temperature and the concentration of the reagents, upper limits of which are however difficult to determine, because the time factor must also be taken into consideration. No such ill effects result from the action of 8% hydrochloric, 5% nitric, and 10% trichloroacetic acids for 48 hours under 35°C.

8. I should like to recommend the lowered-pressure-warming method. By this method, the time required can be reduced to 1/3 to 1/6 of that by the customary method. The Figs. 1, 4, 5, 6, and 7 show microscopic preparations made by this method.

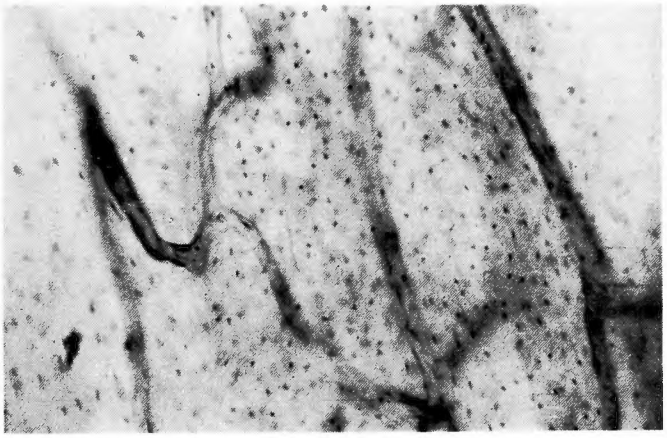
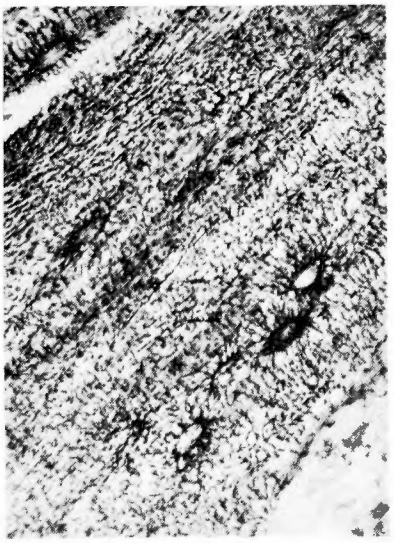


Fig. 1, showing the osseous substance of the femur of a normal adult dog stained by hematoxylin and eosin after 8% trichloroacetic acid decalcification for 48 hours under 1/20 atm. and 30°C. (×200).



Figs. 2 and 3 show the cat's femur stained by BIELSCHOWSKY's method for nerve fibers after E. D. T. A. decalcification for one week. In the osseous substance are found blood vessels, haversian canals, and fibers (nerves?)(Fig. 2; ×40, Fig.3; ×200).

Fig. 1, 5 and 6 show the normal human cranial bones stained by hematoxylin and eosin after 8% trichloroacetic acid decalcification for 48 hours under 1/20 atm. and 30°C.

Fig. 4 At right A. carotis interna is seen obiquely cut at sulcus caroticus, and at left radix of N. trigeminus (proximal to G. Gasseri). The structure beneath them is the sphenoidal bone (×8).

Fig. 5 Sagittal cut of the sphenoidal bone containing Gl. pituitaria, a little left from the median line (×8).

Fig. 6 Higher magnification of Fig. 5 showing osseous substances and bone marrows (×240). Bone cells were observed in the osseous substances, and fat cells in the bone marrow.

Fig. 7 The parietal bone in frontal cut. Blood vessels, haversian canals, and fatty and red marrows are seen (×60)

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和 文 抄 録

骨 脱 灰 法 の 研 究

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横 田 彰

骨及び歯牙等の脱灰に関して今日迄種々な方法及び脱灰液が用いられているが，その各々について追試を行い次の如き結論を得た．

1) 脱灰速度は酸の種類に関係せず，酸度又はpH，脱灰液の温度，液面の圧力に関係する．

2) 数種の酸を混合したり，添加物を加える事は，脱灰速度及び染色性に何等影響を与えない．

3) イオン交換樹脂は本質的には脱灰速度を速めるに役立たないが，脱灰液交換の手間は除ける．

4) 電気的脱灰の効果は全くジュール熱によるものである．

5) E. D. T. A. による脱灰は，神経染色等の酸類使用不可のものに用うべき方法である．

6) 超短波は脱灰速度促進には効果はないと考えられる．

7) 脱灰による組織の破壊と染色性の阻害とは，脱灰液の温度，濃度，及び脱灰に要する時間によつて影響されるが，その条件の決定は困難である．但し大体8％塩酸，5％硝酸，10％三塩化醋酸を用いるなら，48時間以内，35℃以下で組織の破壊及び染色性の阻害は認められない．

8) 1/20気圧30°～35℃加温にて脱灰を行うならば旧来の方法より1/3～1/6の速さで脱灰する事が出来る．